AD_____

Award Number: W81XWH-06-1-0464

TITLE: Effect of MUC1 expression on EGFR endocytosis and degradation in human breast cancer cell lines

PRINCIPAL INVESTIGATOR: Rachid M El Bejjani, M.S.

CONTRACTING ORGANIZATION: The University of Arizona
Tucson, AZ 85722

REPORT DATE: April 2007

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01/04/07	Annual Summary	1 Apr 2006 – 31 Mar 2007

4. TITLE AND SUBTITLE

Effect of MUC1 expression on EGFR endocytosis and degradation in human breast cancer cell lines

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0464

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Rachid M El Bejjani, M.S.

E-Mail: rachid@email.arizona.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Arizona
Tucson, AZ 85722

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: **ErbB receptors are key regulators of cell survival and growth in normal and transformed tissues. The oncogenic glycoprotein MUC1 is a binding partner and substrate for erbB1 and MUC1 expression can potentiate erbB-dependent signal transduction. After receptor activation, erbB1 is typically downregulated via an endocytic pathway that results in receptor degradation or recycling. We report here that MUC1 expression inhibits the degradation of ligand-activated erbB1. Through the use of both RNAi-mediated knock down and overexpression constructs of MUC1, we show that MUC1 expression inhibits erbB1 degradation after ligand treatment in breast epithelial cells. This MUC1-mediated protection against erbB1 degradation can increase total cellular pools of erbB1 over time. Biotinylation of surface proteins demonstrates that cell-surface associated erbB1 receptor is protected by MUC1 against ligand-induced degradation, although this is accompanied by an increase in erbB1 internalization. The MUC1-mediated protection against degradation occurs with a decrease in EGF-stimulated ubiquitination of erbB1, and an increase in erbB1 recycling. These data indicate that MUC1 expression is a potent regulator of erbB1 receptor stability upon activation and may promote transformation through the inhibition of erbB1 degradation.**

15. SUBJECT TERMS
MUC1, EGFR, Endocytosis, Degradation, Ubiquitination, Recycling

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	35	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

	Page
Introduction……..	**4**
Body……..	**4**
Key Research Accomplishments……..	**5**
Reportable Outcomes……	**5**
Conclusion……	**5**
References……	**6**
Appendices……	**7**

INTRODUCTION:

EGFR is a member of the ErbB family of receptor tyrosine kinases. Ligand binding to this receptor induces phosphorylation of the receptor and activation of its kinase domain (1). Activation of the receptor induces signaling that leads to cell growth, proliferation, migration and inhibition of apoptosis. Activated receptors are internalized and in normal cases shuttled from the early endosome to the late endosome and eventually to the lysosome where they are degraded (2). MUC1 is a highly glycosylated membrane protein. It is overexpressed in 90% of breast cancer and has been shown to cause metastatic breast cancer when overexpressed in mouse mammary glands (3). We have shown in our preliminary data that MUC1 and EGFR interact and that MUC1 overexpression inhibits the degradation of activated EGFR in BT20 human breast cancer cell lines. We set out to examine the effect of MUC1 overexpression on the endocytosis and degradation of EGFR. We examine the possibility that MUC1 could be sequestering EGFR in an intracellular compartment or enhancing its recycling to the plasma membrane.

BODY:

In our First task, we proposed to investigate the localization of MUC1 and EGFR (epidermal growth factor receptor) interaction and the effect of MUC1 overexpression on the activation and degradation of EGFR. We have previously shown that MUC1 overexpression inhibits the degradation of EGFR in BT20 human breast cancer cells (see preliminary data*).* We have since shown this same fact in a different breast cancer cell line (MDA-MB-231) and in a normal breast tissue immortalized cell line (MCF10A). We have performed these experiments by overexpressing MUC1 in cells that have low levels of MUC1 expression (MDA-MB-231) and by inhibiting MUC1 expression in cells that naturally overexpress the protein (BT20 and MCF10A) (See attached article Figures 1 and 2 in annexed paper (4)). We then showed that MUC1 affects Cell surface EGFR, We also quantified the average differences from 3 separate experiments and showed consistency in inhibition of surface EGFR degradation in cells that consistently overexpress MUC1. We also showed colocalization of EGFR and MUC1 at the cell membrane in untreated cells and, as shown in our preliminary results, we reported colocalization in perinuclear compartments in cells treated with EGF for 30 min, preliminary data and Figure 3 (4).

In order to understand the mechanism by which MUC1 is inhibiting EGFR degradation, We investigated the effect of MUC1 overexpression on the internalization of EGFR. We found that the overexpression of MUC1 leads to the accelerated internalization of surface EGFR. This was observed using two different methodologies. In our first set of experiments we labeled cell surface proteins by biotinylation after treatment with EGF and incubation for the desired timepoints. We then stopped the biotinylation reaction and lysed the cells. The biotinylated proteins were precipitated on SA-coated beads and the precipitate was probed for EGFR. The levels of EGFR observed reflect the EGFR that remains at the cell surface after EGF stimulation and incubation for a specific timepoint and therefore reflects the effect of MUC1 on the abitlity of EGFR to get internalized upon EGF treatment. We also performed Immunofluorescence analysis of EGFR localization before and after EGF treatment and obtained the same result (see Fig 4 (4)). This is a controversial finding since the internalization of EGFR leads to its transport into the lysosomes and eventually to its degradation. However, these observations correlate with our previous results that indicate that EGFR and MUC1 colocalize in intracellular compartments, preliminary data and Figure 3 (4).

Upon EGF treatment, EGFR is also ubiquitinated by the E3 ubiquitin ligase Cbl (5). This has been shown to be a marker that leads to the sorting of ubiquitinated receptors to the lysosome and ultimately to the degradation of these receptors and to the inhibition of their signaling pathway (6). We assessed the levels of EGF-induced ubiquitination of EGFR in the presence or absence of MUC1 overexpression. We found that upon EGF stimulation, EGFR is highly ubiquitinated in cells that do not overexpress MUC1. MUC1 overexpression in these cells induced a dramatic reduction in EGF-induced EGFR ubiquitination, Figure 5(4). These findings provide a biochemical mechanism for the inhibitory effect of MUC1 on EGFR degradation.

We next set out to determine the fate of the non-ubiquitinated receptors that are not degraded. The logical experiment is to investigate the levels of EGFR recycling to the plasma membrane after EGF treatment in the presence or absence of MUC1. We found that MUC1 overexpressing cells induce a higher level of EGFR recycling 60 and 90 minutes after EGF treatment, Figure6 (4). These results correlate with our findings that

propose a decrease in ubiquitination of EGFR in the presence of MUC1 and explain our finding that MUC1 overexpression induces EGF-dependent EGFR internalization but decreases its degradation.

This implies that MUC1 overexpression induces the internalization of EGFR but inhibits its ubiquination. The internalized receptors are therefore not transported to the lysosome to be degraded and are instead recycled back to the membrane which leads to the availability of higher numbers of EGFR receptors in cells that overexpress MUC1. This would explain the observed inhibition of degradation of EGFR and the potentiation of its activation by MUC1 overexpression.

In our attempt to determine the identity of the intracellular compartment where MUC1 and EGFR colocalize we have found that EGFR does not colocalize with Markers of the ER and the trans-golgi network or with classical markers of the early, recycling or late endosomes (data not shown). We have preformed Immunolocalization studies with proteins that have been shown to be essential for EGFR internalization (Grb2, CALM and Cbl). These studies were performed in BT-20 cells that naturally overexpress MUC1. We have observed colocalization of EGFR and CALM (clathrin assembly lymphoid myeloid leukemia) in perinuclear endocytic compartments. We also observe CALM and MUC1 colocalization. However, we were not able to observe colocalization of MUC1 or EGFR with either Cbl or Grb2. However these studies still need to be confirmed, repeated in different cell lines and the role of MUC1 overexpression remains to be investigated.

We have also started the work on our third aim which proposes the identification of the region of MUC1 that is responsible for the effects on EGFR activation and degradation. We have cloned the constructs discussed in our third aim into the PcDNA3 CMV expression vector and have generated most of the stable transfectants proposed.

KEY RESEARCH ACCOMPLISHMENTS:

- MUC1 inhibits the degradation of EGFR in breast cancer cells and in breast normal epithelial cells
- MUC1 inhibits the degradation of biologically relevant cell surface EGFR
- MUC1 interacts with EGFR at the cell surface and in intracellular compartments
- MUC1 enhances the internalization of EGFR upon EGF stimulation
- MUC1 inhibits the EGF-induced ubiquitination of EGFR
- MUC1 enhances the recycling of EGFR to the plasma membrane

REPORTABLE OUTCOMES:

el Bejjani RM Pochampalli MR, Schroeder JA. MUC1 is a novel regulator of ErbB1 receptor trafficking. Oncogene. 2007 Mar 15;26(12):1693-701. Epub 2006 Sep 18.

Rachid El Bejjani and Joyce A Schroeder. Effect OF MUC1 expression on EGFR endocytosis and degradation in Human breast cancer cell lines (Poster presented at the 25th Congress of the International Association for Breast Cancer Research, Montreal, Canada September 2006)

CONCLUSION:

Our research in the year 2006-2007 led to the publication of our first manuscript annexed to this report, to the ongoing investigation of our proposed aims and to a excellent experience and exposure to international breast cancer research at the 25th congress of the IABCR in Montréal, Canada in September 2006 where I presented a poster depicting our most recent findings and ongoing efforts.

In our recent publication annexed to this report (4), we showed that MUC1 overexpression induces the inhibition of EGFR degradation. We then provided a mechanism for this inhibition of degradation. We showed that MUC1 overexpression affects surface EGFR activation and degradation and that MUC1 accelerates EGFR internalization from the cell surface. We then showed that MUC1 induces a decrease in ubiquination of EGFR and an increase in EGFR recycling to the plasma membrane. This implies that MUC1 increases the internalization of EGFR but since the internalized EGFR is not ubiquitinated, it is recycled back to the membrane instead of being sent to the lysosome to be degraded. This results in an increase in EGFR activation in cells that overexpress MUC1

REFERENCES:

1. Schroeder JA, Lee DC. (1997). Transgenic mice reveal roles for TGFalpha and EGF receptor in mammary gland development and neoplasia. J Mammary Gland Biol Neoplasia 2:119–129.
2. Waterman H, Yarden Y. (2001). Molecular mechanisms underlying endocytosis and sorting of ErbB receptor tyrosine kinases. FEBS Lett 490:142–152.
3. Schroeder JA, Masri AA, Adriance MC, Tessier JC, Kotlarczyk KL, Thompson MC et al. (2004). MUC1 over expression results in mammary gland tumorigenesis and prolonged alveolar differentiation. Oncogene 23: 5739–5747.
4. el Bejjani RM Pochampalli MR, Schroeder JA. MUC1 is a novel regulator of ErbB1 receptor trafficking. Oncogene. 2007 Mar 15;26(12):1693-701. Epub 2006 Sep 18.
5. Levkowitz G, Waterman H, Ettenberg SA, Katz M, Tsygankov AY, Alroy I et al. (1999). Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. Mol Cell 4: 1029–1040.
6. Levkowitz G, Waterman H, Zamir E, Kam Z, Oved S, Langdon WY et al. (1998). c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. Genes Dev 12:3663–3674.

APPENDIX:

MUC1 is a Novel Regulator of ErbB1 Receptor Trafficking

Mamata R. Pochampalli*, Rachid M. el Bejjani*, and Joyce A. Schroeder#

Department of Molecular and Cellular Biology, Arizona Cancer Center and Bio5 Institute, University of Arizona, Tucson Arizona 85724

*These authors contributed equally to this work.

#Corresponding author: jschroeder@azcc.arizona.edu

Key Words: erbB1, MUC1, phosphorylation, ubiquitination, breast cancer

Abstract

ErbB receptors are key regulators of cell survival and growth in normal and transformed tissues. The oncogenic glycoprotein MUC1 is a binding partner and substrate for erbB1 and MUC1 expression can potentiate erbB-dependent signal transduction. After receptor activation, erbB1 is typically downregulated via an endocytic pathway that results in receptor degradation or recycling. We report here that MUC1 expression inhibits the degradation of ligand-activated erbB1. Through the use of both RNAi-mediated knockdown and overexpression constructs of MUC1, we show that MUC1 expression inhibits erbB1 degradation after ligand treatment in breast epithelial cells. This MUC1-mediated protection against erbB1 degradation can increase total cellular pools of erbB1 over time. Biotinylation of surface proteins demonstrates that cell-surface associated erbB1 receptor is protected by MUC1 against ligand-induced degradation, although this is accompanied by an increase in erbB1 internalization. The MUC1-mediated protection against degradation occurs with a decrease in EGF-stimulated ubiquitination of erbB1, and an increase in erbB1 recycling. These data indicate that MUC1 expression is a potent regulator of erbB1 receptor stability upon activation and may promote transformation through the inhibition of erbB1 degradation.

Introduction

The erbB receptor family of tyrosine kinases are frequently deregulated in cancer, and commonly amplified and/or overexpressed in invasive carcinoma [reviewed in (Schroeder & Lee, 1997)]. The family is comprised of four homologous receptors and multiple related ligands. The receptors are type 1 tyrosine kinase transmembrane glycoproteins and include erbB1 (Epidermal Growth Factor Receptor/HER1), erbB2 (HER2/neu), erbB3 (HER3), and erbB 4 (HER4). Ligands for the family include epidermal growth factor (EGF), transforming growth factor alpha (TGFα), amphiregulin (AR), heparin-binding EGF (HB-EGF), betacellulin (BTC), epiregulin (EPR), and epigen [reviewed in (Schroeder & Lee, 1997) and (Strachan et al., 2001)]. Ligand-induced receptor homo- or hetero-dimerization results in tyrosine kinase activation and transphosphorylation of tyrosine residues in the cytoplasmic domain. This leads to the recruitment of a variety of effector proteins including Src, PI 3-kinase, Shc, PLCγ, STATs, Grb2, and cbl, resulting in proliferation, inhibition of apoptosis, differentiation, or degradation of endocytosed receptors (Alroy & Yarden, 1997; Carpenter, 2000; Olayioye et al., 1999; Olayioye et al., 1998).

In addition to activation of signaling cascades at the cell surface, erbB1 also maintains signaling complexes during endocytosis (Waterman & Yarden, 2001). Upon ligand binding, erbB1 becomes bound by the ubiquitin ligase, cbl, and is recruited into clathrin-coated vesicles and internalized (Levkowitz et al., 1999; Waterman & Yarden, 2001). These early endosomes traffic through the cell, eventually maturing into late endosomes and finally delivering their cargo to the lysosome, where the activated receptor is degraded. ErbB1 continues to activate signaling pathways during endosomal trafficking, including Ras and Akt (Bivona & Philips, 2003; Wang et al., 2002). Receptors not targeted for lysosomal degradation are sent to the recycling endosome after releasing their ligand, where they return to the cell surface. It is important to note that degradation of activated receptor is critical to normal regulation of erbB1, and the loss of erbB1 degradation machinery results in transformation (Shtiegman & Yarden, 2003; Thien et al., 2001).

The function of erbB receptors can be modulated by the coexpression of non-erbB transmembrane proteins. One protein shown to modulate the function of erbB1 is the proto-oncogene MUC1 (DF3, CD227,

episialin, PEM)(Schroeder et al., 2004). MUC1 is a heavily O-glycosylated heterodimeric protein of >300 kDa, normally expressed abundantly on the apical surface of glandular epithelia. In greater than 90% of human breast carcinomas and metastases, apical localization is lost and MUC1 is overexpressed (by greater than 10 fold) and underglycosylated (Hilkens et al., 1995; Zotter et al., 1988). O-linked glycosylation of MUC1 occurs through repeated rounds of endocytic recycling via the *trans* Golgi network (Litvinov & Hilkens, 1993). Fully glycosylated MUC1 is maintained through constant endocytosis and recycling (Hanisch & Muller, 2000).

MUC1 expression has been shown to induce transformation in a number of systems, including MMTV-MUC1 transgenic mice and MUC1 transfected 3Y1 rat fibroblasts (Li et al., 2003; Schroeder et al., 2004). In MMTV-MUC1 transgenic mice, tumorigenesis is accompanied by a failure of the mammary gland to undergo complete postlactational regression via apoptosis (Schroeder et al., 2004). Transfection of MUC1 constructs into colon cancer cells demonstrates that MUC1 overexpression inhibits drug-induced apoptosis as well (Ren et al., 2004).

It has been established in both human breast cancer cell lines and transgenic mice overexpressing MUC1 (MMTV-MUC1) that MUC1 and the erbB family of receptor tyrosine kinases biochemically interact (Li et al., 2001b; Schroeder et al., 2001). Importantly, experiments in the MMTV-MUC1 transgenic model have shown that this interaction results in the potentiation of EGF-dependent signaling pathways. Examination of the Ras/MAP Kinase pathway in these transgenic mice demonstrated that overexpression of MUC1 vastly increases EGF-dependent p42/44 ERK activation during lactation (Schroeder et al., 2001). In the present study, we have evaluated the ability of MUC1 expression to inhibit erbB1 degradation as a mechanism of modulating erbB signaling. These studies demonstrate that MUC1 expression inhibits the ligand-mediated ubiquitination and degradation of erbB1 while enhancing its internalization and recycling.

Results

MUC1 expression prolongs EGF-dependent erbB1 phosphorylation

Regulation of erbB1 receptor signaling and its role in transformation are dependent upon the expression levels and duration of activation. Therefore we sought to determine if MUC1 plays a role in either or both of these events by altering the levels of MUC1 expression in breast epithelial cell lines. Three cell lines were chosen for our study, two breast cancer cell lines, BT20 and MDA-MB-231, and one immortalized breast epithelial line, MCF10A. Both BT20 and MCF10A cells express significant levels of MUC1 and erbB1 and were used to analyze the effects of MUC1 knockdown on erbB1 expression in transformed and immortalized conditions, respectively. Parental MDA-MB-231 cells express low levels of MUC1, but similar levels of erbB1 to BT20 cells, and were used to determine the effects of MUC1 overexpression on erbB1 expression and function.

Using RNAi (siRNA), we transiently reduced the expression of MUC1 in BT20 cells (Figure 1A, panel 3). We verified the specificity of our siRNA by demonstrating that the expression of an unrelated protein, β-actin, was unaffected (Figure 1A, panel 4). Furthermore, we verified that these effects were specific to the MUC1 siRNA by using an unrelated control siRNA that had no effect on MUC1 protein expression (Figure 1A, panel 3). The control siRNA sequence was chosen based on its known non-homology to any mammalian genes, and did not result in the alteration in the expression of MUC1, erbB1, or β-actin (see figures 1-4). Finally, we also used 4 additional MUC1 siRNA oligos that gave similar results to those described here to verify specificity (data not shown).

Ligand-bound erbB1 typically dimerizes, becomes internalized into early, then late endosomes, and is either degraded or recycled to the cell surface (Kuwada et al., 1998; Levkowitz et al., 1999; Levkowitz et al., 1998; Waterman & Yarden, 2001). To induce ligand-dependent degradation of erbB receptors, cells transfected with siRNA to MUC1 (BT20-M) or a non-silencing control (BT20-C) were treated with EGF, and endocytosis was allowed to proceed. At times corresponding to receptor activation and internalization (5') and receptor trafficking (15') and degradation (30' or greater), cells were lysed and levels of phosphorylated receptor were analyzed.

Cells that were grown in the presence or absence of serum (but not treated with EGF) were used as controls to distinguish the effects of the growth factors present in serum.

We found that while the total levels of tyrosine-phosphorylated receptor (Figure 1A, top panel) are unchanged in BT20-M at early timepoints (5' and 15'), the same cells showed a significant decrease in phosphorylated erbB receptor at 30'. We continued to monitor the effect of MUC1 expression on erbB stability for 60' and 120' and found that the presence of MUC1 prevented the degradation of phosphorylated erbB receptor for these extended time points. Additionally, we observed decreased levels of total erbB receptor in the same treatment groups indicating total protein loss occurred, instead of loss of receptor phosphorylation (Figure 1A, panel 2).

To determine if this effect was dependent upon active erbB1 kinase, BT20 cells (not treated with RNAi) were treated with the erbB1 kinase inhibitor AG1478 (10μM) 2 hours prior to EGF treatment (Lee et al., 2005; Pai et al., 2002). In these cells, the detection of phosphorylated erbB receptor was ablated by treatment with the kinase inhibitor (Figure 1B). This demonstrated that the observed phosphorylation was dependent upon the kinase activity of erbB1 receptor.

As cells transfected with MUC1 siRNA showed a loss of erbB1 over time, we next determined the long-term effect of changes in MUC1 expression through the transfection and stable selection of either a RNAi hairpin loop vector (pSuper or pSuper-MUC1) or a CMV-MUC1 overexpression construct. To perform these experiments, we utilized either the MCF10A cell line (to knockdown MUC1 expression) or the MDA-MB-231 cell line (to overexpress MUC1). The MDA-MB-231 cells endogenously express very low levels of MUC1 compared to MCF10A cells, but similar levels of erbB1 (Figure 2B).

We first examined if overexpression of MUC1 would stabilize erbB1 expression upon EGF treatment (have the opposite effect to MUC1 knockdown observed in Figure 1A). We stably transfected the MDA-MB-231 cell line with either a CMV-MUC1 (CM) or CMV (C) expression construct (McDermott et al., 2001), which resulted in increased expression of MUC1 (Figure 2A, panel 3). We found that overexpression of MUC1 in these cells stabilized phospho-erbB1 expression in response to EGF treatment as early as 5' after ligand treatment, when phospho-erbB1 begins to be lost in these cells (Figure 2A, panel 1). This inhibition of degradation continued on

and became more pronounced at 30'. Furthermore, after EGF treatment we observed a decrease in the degradation of total erbB1 levels in the presence of MUC1, similar to our observations with the BT20 RNAi experiment (compare Figure 2A, panel 2 to Figure 1A, panel 2). Note that in the absence of serum total levels of erbB1 were unaffected by MUC1 overexpression (Figure 2A).

Finally, we found that stable knockdown of MUC1 expression altered the EGF-induced degradation of phosphorylated erbB1 in MCF10A breast epithelial cells. We used the commercially-available pSuper vectors to express either MUC1 or control sequences that form hairpin loops upon transcription (Brummelkamp et al., 2002). These hairpins are then processed by the cells endogenous machinery to create dsRNA, inducing RNAi-mediated degradation of target sequences. MCF10A cells were transfected with either the pSuper-MUC1 (pSM) or pSuper-Control (pS) vector and selected with neomycin. Stable selection resulted in a significant loss of MUC1 expression in the pSM, while not affecting MUC1 expression in the pS cells (Figure 2C, panel 3). This loss of MUC1 correlates with a significant loss of erbB phosphorylation in response to EGF treatment at the 5' timepoint, although total erbB1 expression is minimally affected at this time. After 45' of treatment with EGF, total erbB1 expression is lost in the absence of MUC1 expression, while erbB1 is still detected in MUC1 expressing cells.

MUC1 expression affects levels of plasma membrane localized erbB1

To determine if the pool of erbB1 that is affected by MUC1 was surface or cytoplasmically derived, we biotinylated cell surface proteins (with cell-impermeable biotin) and performed endocytosis assays to determine the fate of surface-associated erbB1. BT20 cells (either control or treated with MUC1 siRNA) were treated with biotin to label all surface proteins, the biotin reaction was quenched, cells were treated with EGF to induce endocytosis, and biotinylated protein was precipitated with streptavidin beads (Figure 3A, top panel). We found that a reduction of MUC1 expression by RNAi resulted in reduced detection of surface biotinylated erbB1 at the 30' and 60' timepoints. Alternatively, surface biotinylated erbB1 continued to be detected at these timepoints in the presence of MUC1 expression. Densitometry analysis of 3 separate experiments demonstrated an average of ~50% reduction in erbB1 receptor expression in cells treated with MUC1 RNAi compared to controls after 30',

which increased to ~80% after 60' (Figure 3B). Importantly, these data demonstrate that the pool of erbB1 receptors that is affected by MUC1 expression resides at the membrane. Additionally, as we detected total surface erbB1 and its degradation, these data clarify that we detected the degradation of erbB1 protein, and not merely a loss of erbB1 phosphorylation.

We next determined where MUC1 and erbB1 localized in the cell before and after treatment with EGF. To do this, we examined localization of MUC1 and erbB1 in serum starved or EGF treated BT20 cells (without siRNA treatment). As expected, MUC1 and erbB1 colocalized at the cell surface prior to treatment with ligand (Figure 3B left panel, arrow).

MUC1 and erbB1 colocalized in internal compartments upon treatment with EGF for 30', accompanied by an accumulation at distinct intracellular vesicles (Figure 3C, right panel, arrow). These results demonstrate that while MUC1 interacts with erbB1 at the cell surface, the two proteins can be observed in cytoplasmic compartments upon ligand treatment.

MUC1 promotes internalization of erbB1 in response to EGF

Since MUC1 expression inhibits the degradation of plasma membrane-localized erbB1, we next examined if MUC1 expression alters erbB1 retention at the membrane. Stably transfected MDA-MB-231 C and CM cells (described in Figure 2) were serum-starved then treated with EGF to induce internalization (Figure 4A). 5, 30, 60 and 120' after internalization, cell surface proteins were biotinylated, and precipitated with streptavidin. It is important to emphasize that in this experiment, we biotinylated surface proteins after EGF treatment and internalization, a method that would indicate levels of receptor remaining on the surface after ligand-induced internalization. Therefore only those proteins that remained on the cell surface after ligand treatment and internalization (or had returned to the surface through recycling) would be detected. We observed significantly less erbB1 on the cell surface in the presence of MUC1 (after ligand treatment and endocytosis proceeded; Figure 4A), indicating that MUC1 promotes the loss of erbB1 from the cell surface. Increased internalization of erbB1 was strongly enhanced by MUC1 expression at 5' and 30', with total loss of surface erbB1 observed at 60'

regardless of MUC1 expression. Densitometry analysis of 3 separate experiments showed a decrease of surface erbB1 in the presence of MUC1 of ~40% after 5', with total levels of surface erbB1 returning to approximately equal after 2 hours [rel. intensity = 16 (C) vs 10 (CM) at 5'; and 16 (C) vs 14 (CM) at 2 hours]. Note that we are comparing relative levels of C to CM at these timepoints.

Analysis of erbB1 localization by immunofluorescence in stably transfected MDA-MB-231 cells (both CM and C) visually recapitulated this observation. In the absence of serum, erbB1 is found at the plasma membrane regardless of MUC1 expression (Figure 4B, top panels). Alternatively, the MUC1 overexpressing cells (CM) display significant internalized erbB1, with very little surface erbB1 visible upon 30' of EGF treatment (Figure 4B, right panel). Alternatively, while the control (C) cells demonstrated internalized erbB1 in response to EGF treatment, there were still significant levels of erbB1 observed on the surface (Figure 4B, left panel). This data again indicates that in those cells expressing high levels of MUC1, erbB1 internalization was enhanced. Together, these results demonstrate that, although MUC1 inhibits the degradation of ligand-activated erbB1, it also promotes the internalization of the receptor.

MUC1 expression inhibits the ubiquitination of erbB1

To determine the mechanism of MUC1-mediated inhibition of erbB1 degradation, we examined the ubiquitination of erbB1 in response to EGF treatment. Ligand-bound erbB1 is normally ubiquitinated (ub-erbB1) upon internalization and vesicular trafficking then proceeds [reviewed by (Waterman & Yarden, 2001)]. We treated either MDA-MB-231C or CM cells with serum alone or EGF for 2' at 37°C to induce internalization and ubiquitination. While ub-erbB1 was observed with this treatment in control cells (C), ub-erbB1 was markedly reduced in CM cells (Figure 5A). Also, while ub-erbB1 was not observed in the presence of serum at the exposure times required to detect EGF-induced ub-erbB1, we did observe ub-erbB1 in the presence of serum at longer exposure times (Figure 5B). This ub-erbB1 was also reduced in the presence of MUC1 expression, indicating that the growth factors present in serum can also induce (albeit much less) ubiquitination of erbB1.

These data demonstrate that MUC1 expression alters the ubiquitination of erbB1, indicating a mechanism for the decrease in erbB1 degradation.

MUC1 expression increases erbB1 recycling

To determine if the decrease in erbB1 ubiquitination was due to altered erbB1 trafficking, we examined erbB1 recycling to the plasma membrane. MDA-MB-231C and CM cells were pulsed with EGF for 10' on ice and unbound EGF was then washed off. Treated cells were then chased by incubating at 37°C for 10, 60 and 90' before being harvested and incubated with an erbB1-PE antibody (Kowanetz et al., 2004; Reinheckel et al., 2005; Yan et al., 2005).

We detected an ~80% increase in the amount of recycled erbB1 in CM cells compared to C cells 60' after EGF stimulation, and an approximate 40% increase in recycling 90' after stimulation (Figure 6). Recycling is shown as the % difference between the average corrected geometric mean at each experimental time point (TP) minus the starting (10') timepoint [(TP-T10')/T10'*100], and represents the amount of erbB1 that is recycled to the cell surface after each time analyzed. These results indicate that MUC1 overexpression promotes the recycling of erbB1 to the cell surface, providing a mechanism by which MUC1 inhibits erbB1 degradation and promotes erbB1 signaling.

Discussion

We report here that MUC1 expression inhibits the degradation of ligand-activated erbB1 receptor. We show through RNAi-mediated loss of MUC1 expression that MUC1 inhibits the degradation of phosphorylated erbB1 after ligand binding in both BT20 breast cancer cells and MCF10A breast epithelial cells. Overexpression of MUC1 in MDA-MB-231 cells recapitulates this effect by stabilizing ligand-activated erbB receptor. Biotinylation of surface proteins demonstrates that MUC1 promotes the internalization of cell-surface associated erbB1 while protecting it against ligand-activated degradation. Importantly, MUC1 expression inhibits the ubiquitination of erbB1 and enhances its recycling to the plasma membrane.

MUC1 has been previously shown to both interact with erbB receptors (Li et al., 2001a; Schroeder et al., 2001) and affect EGF-dependent activation of MAP Kinase pathways in lactating mammary glands (Schroeder et al., 2001). The mechanism by which MUC1 accomplishes this task, though, was undetermined. ErbB receptors can be regulated both by their activation [i.e. through ligand binding or as a substrate for src kinase (Ishizawar & Parsons, 2004)] and through downregulation of the receptor. Work involving the ubiquitin ligase cbl has shown that downregulation of erbB1 is a critical component of its normal function, and degradation of erbB1 is a key component in preventing it to act in an oncogenic manner (Shtiegman & Yarden, 2003; Thien et al., 2001). Our current data now defines MUC1 as another important regulator of erbB1 receptor degradation. By examining both overexpression constructs and RNAi knockdowns of MUC1, we have demonstrated a functional role for MUC1 as a regulator of erbB1 signaling. We found that CM cells mimicked pS cells (both expressing high MUC1 and displaying stable erbB1 expression) and C cells, in turn, mimicked pSM cells (both expressing very low MUC1 and displaying accelerated erbB1 degradation in response to ligand). These experiments show that both overexpression of MUC1 in low-MUC1-expressing cells or loss of expression in high-MUC1-expressing cells can modulate the stability of phosphorylated erbB1 expression. While a previous study reported that MUC1 expression affects the transcription of erbB1 (Li et al., 2005), we were unable to detect alterations of the steady state level of erbB1 levels in response to MUC1 loss or overexpression. It will be interesting in future work to evaluate the effect of MUC1 expression on the ligand-induced degradation of all members of the erbB family. Given that MUC1 and erbB receptors are commonly overexpressed in breast cancer [reviewed in (Gendler, 2001; Schroeder & Lee, 1997)], the interactions between these proteins may be potent inducers of carcinoma in the appropriate molecular setting.

Our data demonstrate that although MUC1 inhibits ligand-induced degradation of erbB1, it also promotes its internalization. This apparent paradox may be explained by one of two non-exclusive hypotheses. The first is that MUC1 promotes the entry of erbB1 into a recycling pathway instead of an ubiquitination pathway. Our data demonstrate that MUC1 expression does, in fact, inhibit the ubiquitination of erbB1 concurrently with an increase in the recycling of erbB1 to the plasma membrane. Additionally, MUC1 expression could also be driving erbB1

into an alternate internal trafficking pathway, one that does not lead to protein degradation. This could potentially involve trafficking to the ER or Trans Golgi Network, both of which have been shown to alter the degradation of ligand-activated erbB1 (Haj et al., 2002; Puertollano & Bonifacino, 2004). We are currently examining potential mechanisms of alternate trafficking.

Our data indicate that MUC1 alters ligand-induced internalization and degradation of erbB1. While MUC1 promotes internalization of erbB1, MUC1 also inhibits the degradation of erbB1 that would normally follow. Importantly, we have found that MUC1 is altering the normal ubiquitination of internalized erbB1 and promoting the recycling of erbB1 to the cell surface. This study implicates MUC1 as a potentially critical mediator of erbB1 stability and function, one that may have dramatic implications for erbB1-mediated breast cancer progression.

Materials and Methods

Cell lines

BT20 and MDA-MB-231 human breast cancer cells and MCF10A immortalized human mammary epithelial cells were purchased from ATCC and maintained in RPMI (Gibco) with 10% FBS (Biomeda) and 1.0 % Penicillin-streptomycin (Gibco) in 5.0% CO_2 at 37°C. Growth medium for MCF10A cells was supplemented with 10ng/ml cholera toxin (Sigma), 0.5µg/ml hydrocortisone (Sigma) and 5ng/ml EGF (Invitrogen). Cells were grown to 80% confluency for use.

Antibodies, growth factors and cDNA constructs

CT2 (MUC1 cytoplasmic domain) and erbB1 Ab-1 and Ab-21 were purchased from Neomarkers Inc. erbB1 1005 and PY99 were purchased from Santa Cruz Biotechnologies. The DF3 antibody (MUC1 extracellular domain) was purchased from DAKO Corporation. Ubiquitin (p4d1) was from Santa Cruz Biochemicals and NCL-Ubiq was from Novocastra. Antibodies to detect β-actin is from Sigma Chemical Company. Secondary antibodies conjugated to HRP were purchased from Pierce and the HRP-conjugated

Hamster antibody was purchased from Jackson Laboratories. All Alexa-conjugated antibodies were purchased from Molecular Probes (Invitrogen). ErbB1 kinase inhibitor (AG1478) was obtained from (Sigma). The MUC1 cDNA was a kind gift from M. A. Hollingsworth at the Eppley Cancer Institute, University of Nebraska Medical Center. The MUC1 cDNA was subcloned into the pCMV-DNA3.1 vector (Invitrogen) using standard techniques and constructs were transfected into MDA-MB-231 cells using Lipofectamine 2000, and selected with 1mg/ml G418.

RNAi

RNAi was performed by either transient siRNA treatment (Qiagen or Dharmacon) or stable selection of pSUPER hairpin loop vectors (OligoEngine).

siRNA: MUC1 specific siRNA was generated to the following target sequence of the MUC1 extracellular domain: ^{3104}AAGACTGATGCCAGTAGCACT (Lan et al., 1990), and a non-silencing siRNA was designed to the following target sequence, which lacks homology to any known mammalian gene: AATTCTCCGAACGTGTCACGT (Qiagen). Transfections were performed with Lipofectamine 2000 (Invitrogen) following the manufacturer's suggestions. For MCF10A cells, the transfection was performed once and the cells were lysed on day 3 post-transfection. BT20 cells required a double transfection for complete knockdown. The cells were transfected on day 1 then transfected again on day 3. BT20 lysates were collected on day 5. Knockdown was verified by immunoblotting for protein expression.

Additional controls were utilized to verify the effects of the Qiagen manufactured siRNA by testing MUC1 siRNA oligos from a second company (Dharmacon, *SMART*pool of 4 siRNA oligos) on these assays. These target sequences were as follows [and lie in the extracellular (#1-3) and cytoplasmic domain (#4)]: (#1)^{3270}ACCAAGAGCTGCAGAGAGA, (#2)^{3363}GATCTGTGGTGGTACAATT, (#3)^{3465}GATATAACCTGACGATCTC, (#4)^{3746}GATCGTAGCCCCTATGAGA (Lan et al., 1990). Those studies were performed following the manufacturer's instructions.

pSuper: pSuper vectors were purchased from (OligoEngine), using the companies software to generate both MUC1 and control RNAi hairpin loops. The following extracellular target sequence was used for the MUC1 hairpin loop: ^{3073}TACTCCTACCACCCTTGCC (Lan et al., 1990). Cells were transfected using Lipofectamine 2000 (Invitrogen), selected using G418 (Invitrogen) and were continually grown under 250 μg/ml during experiments.

Endocytosis assays

Cells were serum starved overnight, then incubated with 20ng/ml receptor grade EGF (Invitrogen) for 10' on ice. Unbound EGF was then removed by washing 2X with PBS at 4°C. Cells in serum-free media were incubated at 37°C for the indicated time points and lysed [20 mM HEPES pH 7.5, 150 mM NaCl, 2mM EDTA pH 8.0, 2mM EGTA pH 8.0 and 1.0% Triton X-100, 2.0 mM Sodium orthovanadate, 50.0 μM ammonium molybdate and 10.0 mM sodium fluoride and Complete protease inhibitors (Roche)]. Cell lysates were vortexed briefly, centrifuged and the supernatant stored at -80°C. Protein concentrations were determined by BCA assay (Pierce).

Biotinylation assays

BT20 or MDA-MB-231 (C or CM) cells were grown serum-free overnight, then incubated at 4°C with 4 ml of 0.3mg/ml Sulfo-NHS-SS-biotin (Pierce) for 30' to analyze receptor internalization (Figure 3A). The biotinylation reaction was stopped by washing 3X with ice cold quenching buffer (10mM Tris pH 7.4, 154mM NaCl). Cells were then washed with ice cold PBS and treated with EGF as described in the endocytosis assay. For analysis of surface retention (Figure 4A), cells were first treated with EGF and incubated for 5' at 37°C to promote internalization, then treated with biotin and lysed as described above. Lysates were precipitated with streptavidin coated agarose beads, washed and resuspended in SDS-PAGE buffer (0.4M Tris, 0.2mM EDTA, 25% glycerol, 10% β-mercaptoethanol and 0.4% bromophenol blue) for immunoblotting.

Immunofluorescence analysis

Cells were grown on glass coverslips and serum starved overnight. Cells were treated with EGF as described for the endocytosis experiment, washed with 0.02% NaN_3 /PBS, then fixed with a 1:1 mixture of ice cold methanol-acetone. Cells were blocked with 20% FBS/0.02% NaN_3 /PBS, incubated with the indicated antibody overnight at 4°C, washed, and then incubated with Alexa fluor-conjugated secondary antibodies. Cells were then mounted with Slowfade Antifade reagent with DAPI (Molecular Probes) and visualized using a Leica DMBL 100s system with MagnaFire software.

Immunoprecipitation and immunoblotting

Protein lysates were incubated with their respective antibodies in TNEN buffer (50mM Tris pH 7.5, 150 mM NaCl, 1mM EDTA pH 8.0 and 0.5% Igepal CA 630, 2.0 mM Sodium orthovanadate, 50.0 µM ammonium molybdate and 10.0 mM sodium fluoride and Complete protease inhibitors) and rProtein G agarose beads (Invitrogen). Precipitates were washed with TNEN buffer 3X, and proteins were resuspended in 2X SDS PAGE buffer. Proteins were then separated by SDS-PAGE and transferred onto PVDF membrane (Millipore). The membrane was blocked with either 5% nonfat milk in PBS (0.1% Tween20) or 3% BSA in TBS (0.1% Tween20) and immunoblotted. The membrane was then treated with Super Signal West Pico Chemiluminescent Substrate (Pierce), visualized on Imagetech-B film (American X-ray supply Inc), and developed with a Konica SRX-101C.

For ubiquitination experiments, TNEN buffer also contained 10mM N-ethyl-maleimide (Pierce) to inhibit deubiquitination.

Recycling Assay

MDA-MB-231 cells transfected with MUC1 or with PcDNA3 alone were plated in triplicates and treated with 10ng/ml EGF at 4°C for 10'. Unbound EGF was removed by washing (2X) with cold PBS, cells were incubated at 37°C for 10', 60', or 90', trypsinized and blocked with 5% BSA for 1h on ice. The cells were then incubated with R-phytoerythrin (R-PE) conjugated erbB1 antibody (BD Pharmingen San Diego CA) on ice. Fluorescence intensity was then immediately determined on a FACScan flow cytometer (BD biosciences, San Jose CA) and the results were analyzed using a CellQuestPro 4.0 software (BD biosciences, San Jose CA) using standard methodology followed by the Arizona Cancer Center Flow Cytometry Shared Service (FCSS). The geometric mean of stained cells was corrected against non stained cells to eliminate any autofluorescence and the results were plotted as a % difference where the 10' time point was considered the time point of origin at which no recycling occurs. [(TP-T10')/T10'*100]

Acknowledgments

We are grateful to Jean Wilson for helpful suggestions, Todd Camenisch, Jose Lopez, Jeanne Louderbough, Wendy Knowlton and Andrew Chang for a critical reading of this manuscript, and M.A. Hollingsworth for the MUC1 expression vector. FACS analysis was performed by the Flow Cytometry Shared Service of the Arizona Cancer Center. This work was supported by grants from the National Cancer Institute (J.A.S.), the Susan Komen Breast Cancer Foundation (M.R.P.), and the Department of Defense (R.M.B).

References

Alroy, I. & Yarden, Y. (1997). The ErbB signaling network in embryogenesis and oncogenesis: signal diversification through combinatorial ligand-receptor interactions. *FEBS Lett*, **410:** 83-6.

Bivona, T.G. & Philips, M.R. (2003). Ras pathway signaling on endomembranes. *Curr Opin Cell Biol*, **15:** 136-42.

Brummelkamp, T.R., Bernards, R. & Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science*, **296:** 550-3.

Carpenter, G. (2000). The EGF receptor: a nexus for trafficking and signaling. *Bioessays*, **22:** 697-707.

Gendler, S.J. (2001). MUC1, the renaissance molecule. *J Mammary Gland Biol Neoplasia*, **6:** 339-53.

Haj, F.G., Verveer, P.J., Squire, A., Neel, B.G. & Bastiaens, P.I. (2002). Imaging sites of receptor dephosphorylation by PTP1B on the surface of the endoplasmic reticulum. *Science*, **295:** 1708-11.

Hanisch, F.G. & Muller, S. (2000). MUC1: the polymorphic appearance of a human mucin. *Glycobiology*, **10:** 439-49.

Hilkens, J., Vos, H.L., Wesseling, J., Boer, M., Storm, J., van der Valk, S., Calafat, J. & Patriarca, C. (1995). Is episialin/MUC1 involved in breast cancer progression? *Cancer Lett*, **90:** 27-33.

Ishizawar, R. & Parsons, S.J. (2004). c-Src and cooperating partners in human cancer. *Cancer Cell*, **6:** 209-14.

Kowanetz, K., Husnjak, K., Holler, D., Kowanetz, M., Soubeyran, P., Hirsch, D., Schmidt, M.H., Pavelic, K., De Camilli, P., Randazzo, P.A. & Dikic, I. (2004). CIN85 associates with multiple effectors controlling intracellular trafficking of epidermal growth factor receptors. *Mol Biol Cell*, **15:** 3155-66.

Kuwada, S.K., Lund, K.A., Li, X.F., Cliften, P., Amsler, K., Opresko, L.K. & Wiley, H.S. (1998). Differential signaling and regulation of apical vs. basolateral EGFR in polarized epithelial cells. *Am J Physiol*, **275:** C1419-28.

Lan, M.S., Batra, S.K., Qi, W.N., Metzgar, R.S. & Hollingsworth, M.A. (1990). Cloning and sequencing of a human pancreatic tumor mucin cDNA. *J Biol Chem*, **265:** 15294-9.

Lee, F.T., Mountain, A.J., Kelly, M.P., Hall, C., Rigopoulos, A., Johns, T.G., Smyth, F.E., Brechbiel, M.W., Nice, E.C., Burgess, A.W. & Scott, A.M. (2005). Enhanced efficacy of radioimmunotherapy with 90Y-

CHX-A"-DTPA-hu3S193 by inhibition of epidermal growth factor receptor (EGFR) signaling with EGFR tyrosine kinase inhibitor AG1478. *Clin Cancer Res*, **11:** 7080s-7086s.

Levkowitz, G., Waterman, H., Ettenberg, S.A., Katz, M., Tsygankov, A.Y., Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A., Lipkowitz, S. & Yarden, Y. (1999). Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. *Mol Cell*, **4:** 1029-40.

Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W.Y., Beguinot, L., Geiger, B. & Yarden, Y. (1998). c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev*, **12:** 3663-74.

Li, X., Wang, L., Nunes, D.P., Troxler, R.F. & Offner, G.D. (2005). Suppression of MUC1 synthesis downregulates expression of the epidermal growth factor receptor. *Cancer Biol Ther*, **4:** 968-73.

Li, Y., Kuwahara, H., Ren, J., Wen, G. & Kufe, D. (2001a). The c-Src tyrosine kinase regulates signaling of the human DF3/MUC1 carcinoma-associated antigen with GSK3 beta and beta-catenin. *J Biol Chem*, **276:** 6061-4.

Li, Y., Liu, D., Chen, D., Kharbanda, S. & Kufe, D. (2003). Human DF3/MUC1 carcinoma-associated protein functions as an oncogene. *Oncogene*, **22:** 6107-10.

Li, Y., Ren, J., Yu, W., Li, Q., Kuwahara, H., Yin, L., Carraway, K.L., 3rd & Kufe, D. (2001b). The epidermal growth factor receptor regulates interaction of the human DF3/MUC1 carcinoma antigen with c-Src and beta-catenin. *J Biol Chem*, **276:** 35239-42.

Litvinov, S.V. & Hilkens, J. (1993). The epithelial sialomucin, episialin, is sialylated during recycling. *J Biol Chem*, **268:** 21364-71.

McDermott, K.M., Crocker, P.R., Harris, A., Burdick, M.D., Hinoda, Y., Hayashi, T., Imai, K. & Hollingsworth, M.A. (2001). Overexpression of MUC1 reconfigures the binding properties of tumor cells. *Int J Cancer*, **94:** 783-91.

Olayioye, M.A., Beuvink, I., Horsch, K., Daly, J.M. & Hynes, N.E. (1999). ErbB receptor-induced activation of stat transcription factors is mediated by Src tyrosine kinases. *J Biol Chem*, **274:** 17209-18.

Olayioye, M.A., Graus-Porta, D., Beerli, R.R., Rohrer, J., Gay, B. & Hynes, N.E. (1998). ErbB-1 and ErbB-2 acquire distinct signaling properties dependent upon their dimerization partner. *Mol Cell Biol*, **18:** 5042-51.

Pai, R., Soreghan, B., Szabo, I.L., Pavelka, M., Baatar, D. & Tarnawski, A.S. (2002). Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. *Nat Med*, **8:** 289-93.

Puertollano, R. & Bonifacino, J.S. (2004). Interactions of GGA3 with the ubiquitin sorting machinery. *Nat Cell Biol*, **6:** 244-51.

Reinheckel, T., Hagemann, S., Dollwet-Mack, S., Martinez, E., Lohmuller, T., Zlatkovic, G., Tobin, D.J., Maas-Szabowski, N. & Peters, C. (2005). The lysosomal cysteine protease cathepsin L regulates keratinocyte proliferation by control of growth factor recycling. *J Cell Sci*, **118:** 3387-95.

Ren, J., Agata, N., Chen, D., Li, Y., Yu, W.H., Huang, L., Raina, D., Chen, W., Kharbanda, S. & Kufe, D. (2004). Human MUC1 carcinoma-associated protein confers resistance to genotoxic anticancer agents. *Cancer Cell*, **5:** 163-75.

Schroeder, J.A. & Lee, D.C. (1997). Transgenic mice reveal roles for TGFalpha and EGF receptor in mammary gland development and neoplasia. *J Mammary Gland Biol Neoplasia*, **2:** 119-29.

Schroeder, J.A., Masri, A.A., Adriance, M.C., Tessier, J.C., Kotlarczyk, K.L., Thompson, M.C. & Gendler, S.J. (2004). MUC1 overexpression results in mammary gland tumorigenesis and prolonged alveolar differentiation. *Oncogene*, **23:** 5739-47.

Schroeder, J.A., Thompson, M.C., Gardner, M.M. & Gendler, S.J. (2001). Transgenic MUC1 interacts with epidermal growth factor receptor and correlates with mitogen-activated protein kinase activation in the mouse mammary gland. *J Biol Chem*, **276:** 13057-64.

Shtiegman, K. & Yarden, Y. (2003). The role of ubiquitylation in signaling by growth factors: implications to cancer. *Semin Cancer Biol*, **13:** 29-40.

Strachan, L., Murison, J.G., Prestidge, R.L., Sleeman, M.A., Watson, J.D. & Kumble, K.D. (2001). Cloning and biological activity of epigen, a novel member of the epidermal growth factor superfamily. *J Biol Chem*, **276:** 18265-71.

Thien, C.B., Walker, F. & Langdon, W.Y. (2001). RING finger mutations that abolish c-Cbl-directed polyubiquitination and downregulation of the EGF receptor are insufficient for cell transformation. *Mol Cell*, **7:** 355-65.

Wang, Y., Pennock, S., Chen, X. & Wang, Z. (2002). Endosomal signaling of epidermal growth factor receptor stimulates signal transduction pathways leading to cell survival. *Mol Cell Biol*, **22:** 7279-90.

Waterman, H. & Yarden, Y. (2001). Molecular mechanisms underlying endocytosis and sorting of ErbB receptor tyrosine kinases. *FEBS Lett*, **490:** 142-52.

Yan, Q., Hunt, P.R., Frelin, L., Vida, T.A., Pevsner, J. & Bean, A.J. (2005). mVps24p functions in EGF receptor sorting/trafficking from the early endosome. *Exp Cell Res*, **304:** 265-73.

Zotter, S., Hageman, P.C., Lossnitzer, A., Mooi, W.J. & Hilgers, J. (1988). Tissue and tumor distribution of human polymorphic epithelial mucin. *Cancer Reviews*, **11-12:** 55-101.

Figure Legends

Figure 1. MUC1 inhibits the degradation of EGF-stimulated erbB1. A. BT20 cells were transfected with either MUC1 siRNA or control (nonsilencing) siRNA, and either left in normal growth conditions (+ serum), serum starved overnight (- serum), or serum starved overnight and treated with 20 ng/ml EGF for the indicated times prior to lysis. Protein lysates (25 μg) were separated and immunoblotted with antibodies to detect phosphotyrosine (PY99), erbB1 (1005), MUC1 (CT2) or β-actin. B. BT20 cells were serum-starved overnight, treated with 10 μM AG1478 for 2 hours prior to treatment with 20 ng/ml EGF for 10' on ice. Cells were then washed with PBS and incubated at 37°C for the indicated times and lysed. Proteins were then immunoblotted with either anti-erbB1 (1005) or anti-phosphotyrosine (PY99) antibodies. Note: Human MUC1 separates into multiple, differentially glycosylated and phosphorylated sizes (and the cytosplasmic tail that reacts to CT2 is ~14-35 kDa). Molecular weights are indicated on the right.

Figure 2. Stable alteration of MUC1 expression affects erbB1 degradation. A. MDA-MB-231 cells were transfected with either CMV-MUC1 (CM) or parental CMV (C) driven vectors, then stably selected with neomycin. Cell lines were serum starved overnight, then treated with 20ng/ml EGF for 10' at 4°C and washed with PBS to remove unbound ligand. Cells were then incubated for the indicated times at 37°C and lysed. Protein lysates were separated by SDS-PAGE and immunoblotted with either anti-phosphotyrosine (PY99), anti-erbB1 (1005), or anti-MUC1 (CT2) antibodies. B. Protein lysates from parental MCF10A and MDA-MB-231 were made, and 40μg were separated by SDS-PAGE. Relative levels of erbB1 and MUC1 expression in each cell line were determined by immunoblotting with anti-erbB1 (1005) and anti-MUC1 (CT2) antibodies, respectively. C. MCF10A cells were transfected with either pSuper-Control (pS) or pSuper-MUC1 (pSM) RNAi constructs, then stably selected with neomycin. Cells were then treated and immuoblotted as described above in (A). Molecular weights are indicated on the right.

Figure 3. MUC1 expression alters cell-surface erbB1. A. BT20 cells were transfected with either MUC1 siRNA or control (nonsilencing) siRNA. After knockdown, cells were biotinylated at 4°C to label cell surface receptors, and the reaction was quenched with a Tris buffer. After washing, cells were treated with EGF for the indicated timepoints, lysed and protein was precipitated using streptavidin beads (SAP). Proteins were then immunoblotted with anti-erbB1 (1005). Total cell lysates were also separated to demonstrate total cellular levels of protein and immunoblotted with anti-erbB1 (1005) or anti-MUC1 (CT2), bottom 2 panels. NB = Non-biotinylated control. Molecular weights are indicated on the right. B. Quantification of the differences observed in A. Densitometry from 3 separate experiments was obtained using the ScionImage software, corrected against background and averaged. Striped bars represent control-RNAi treated cells and dotted bars represent MUC1-RNAi treated cells C. Left panel: To analyze surface colocalization, BT20 cells were serum-starved overnight. Cells were then labeled with anti-erbB1 (Ab-21) and anti-MUC1 (DF3) for the primary antibodies and anti-rabbit Alexa 594/red and anti-mouse Alexa 488 for the secondaries. (MUC1= green, erbB1= red) Right panel: To analyze internal colocalization during EGF treatment, BT20 cells were serum-starved overnight, then incubated with 20ng/ml EGF for 5 minutes, washed and incubated at 37°C for 30'. Cells were labeled with anti-erbB1 (Ab-1) and anti-MUC1 (CT2) for the primary antibodies and anti-rabbit Alexa 594/red and anti-armenian hamster FITC for the secondaries. Magnification is 400X. Arrows indicate areas of colocalization.

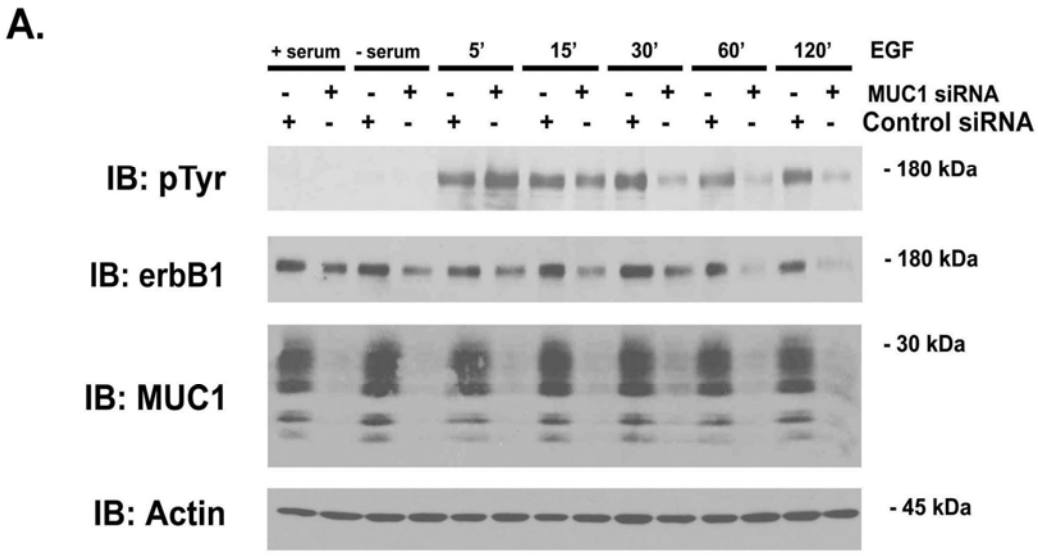
Figure 4. MUC1 expression promotes EGF-dependent internalization. A. To assess receptor internalization, MDA-MB-231 breast cancer cells stably transfected with MUC1 (CM) or with vector alone (C) were treated with 100ng/ml EGF at 4°C (to saturate erbB1 receptors) and incubated for the indicated time point before being surface biotinylated and then extracted. Biotinylated proteins were then precipitated with streptavidin (SAP) and immunoblotted with anti-erbB1 (1005). A separate set of protein lysates were treated and prepared as described and analyzed for MUC1 expression using anti-MUC1 (CT2), bottom panel, under separation line. B. Immunofluorescence of erbB1 in MDA-MB-231 cells expressing either the CMV vector (C) or CMV-MUC1 (CM), serum starved (top panels) or serum starved and treated with 10 ng/ml EGF on ice,

washed, then incubated for 30' at 37°C (bottom panels). Cells were then fixed with methanol/acetone and stained with antibodies against erbB1 (Ab-1), magnification = 630X.

Figure 5. MUC1 expression inhibits the ubiquitination of erbB1. A. MDA-MB-231 cells overexpressing CMV-MUC1 (CM) or the CMV vector (C) were serum starved overnight and treated with 20ng/ml EGF at 37°C for 2' or grown in serum and lysed (+ ser). Protein lysates (500ug) were immunoprecipitated with anti-erbB1 antibody (Ab-1) or control mouse IgG and immunoblotted with an anti-ubiquitin antibody (Santa Cruz). Protein lysates (50ug) from these samples were also immunoblotted with anti-MUC1 (CT2) or anti-erbB1 (Santa Cruz, 1005) to show total levels of protein (bottom two panels). B. MDA-MB-231 cells overexpressing CMV-MUC1 (CM) or the CMV vector (C) were grown in serum and lysed. Protein lysates (300ug) were immunoprecipitated with anti-ubiquitin antibody (NCL-ubiq) or control rabbit IgG and immunoblotted with anti-erbB1 antibody (Santa Cruz 1005). Protein lysates (50ug) from these samples were also immunoblotted with anti-MUC1 (CT2) or anti-erbB1 (Santa Cruz, 1005) to show total levels of protein (bottom two panels). Note that detection of ubiquitinated erbB1 in A was obtained with an exposure time of 30 seconds (using DuraSignal, Pierce Chemical Co.), while the detection of ubiquitinated erbB1 in B was obtained with an overnight exposure (using DuraSignal, Pierce Chemical Co.).

Figure 6. MUC1 over-expression promotes the recycling of erbB1. MDA-MB-231 cells expressing CMV-MUC1 (MUC1/dashed line/squares) or CMV (CMV/solid line/diamond) were pulsed with 10ng/ml EGF for 10' on ice and then incubated at 37°C to induce internalization, and cells were harvested at either 60' or 90'. The mean fluorescence intensity (MFI) was measured by flow cytometry, and the MFI at 10' after EGF stimulation was considered a reference point at which the activated receptors are internalized but not yet recycled. Recycling is represented as the % difference between the MFI at the indicated time point and the MFI at the 10' reference time point.

% recycling = [(TP-T10')/T10'*100].


A.
+ serum
- serum
5'
15'
30'
60'
120'
EGF
MUC1 siRNA
Control siRNA
IB: pTyr
- 180 kDa
IB: erbB1
- 180 kDa
IB: MUC1
- 30 kDa
IB: Actin
- 45 kDa

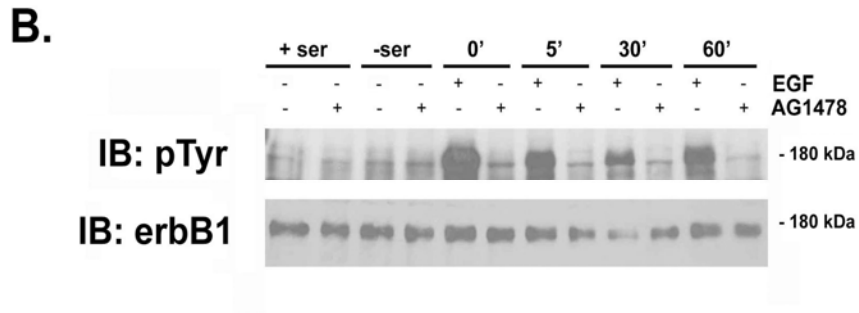

B.
+ ser
-ser
0'
5'
30'
60'
EGF
AG1478
IB: pTyr
- 180 kDa
IB: erbB1
- 180 kDa

FIGURE 1

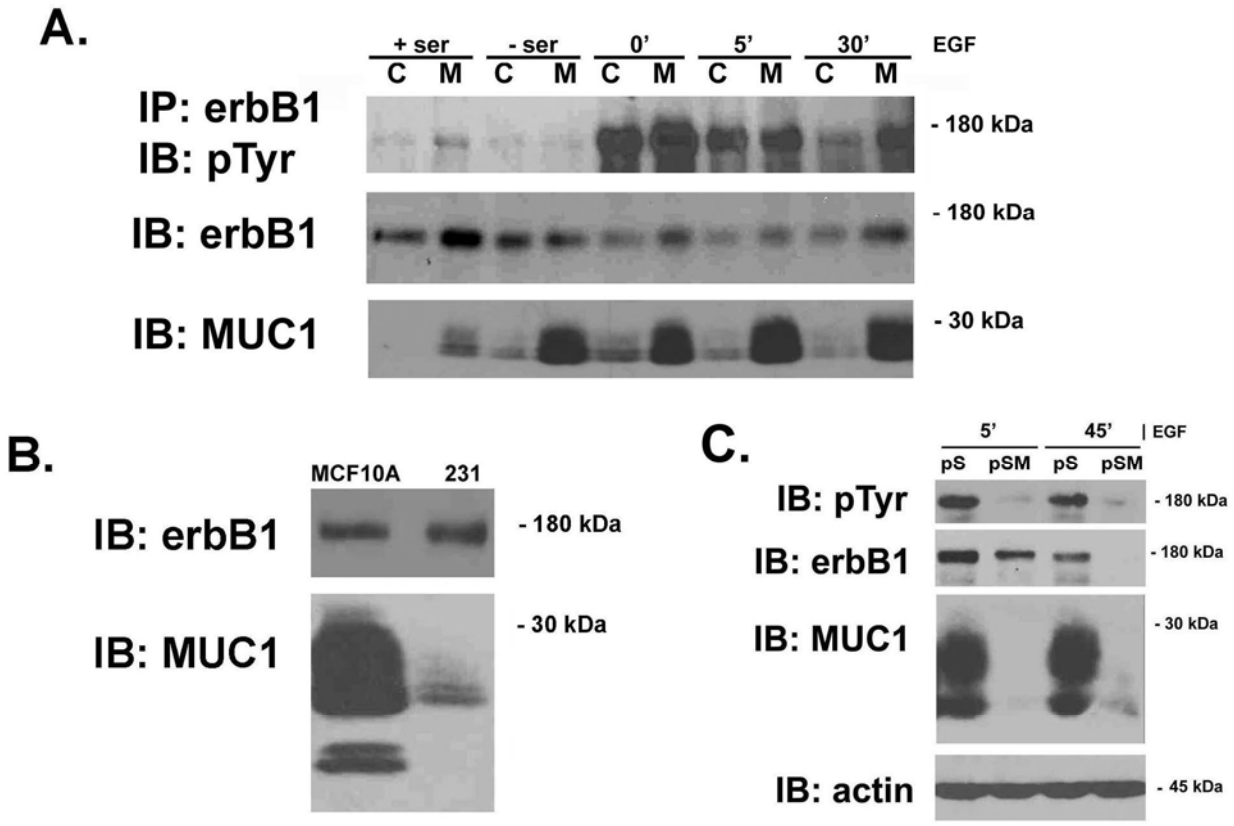

A.
+ ser
- ser
0'
5'
30'
EGF
C M C M C M C M C M
IP: erbB1
IB: pTyr
- 180 kDa
IB: erbB1
- 180 kDa
IB: MUC1
- 30 kDa
B.
MCF10A
231
IB: erbB1
- 180 kDa
IB: MUC1
- 30 kDa
C.
5'
45'
EGF
pS pSM pS pSM
IB: pTyr
- 180 kDa
IB: erbB1
- 180 kDa
IB: MUC1
- 30 kDa
IB: actin
- 45 kDa

FIGURE 2

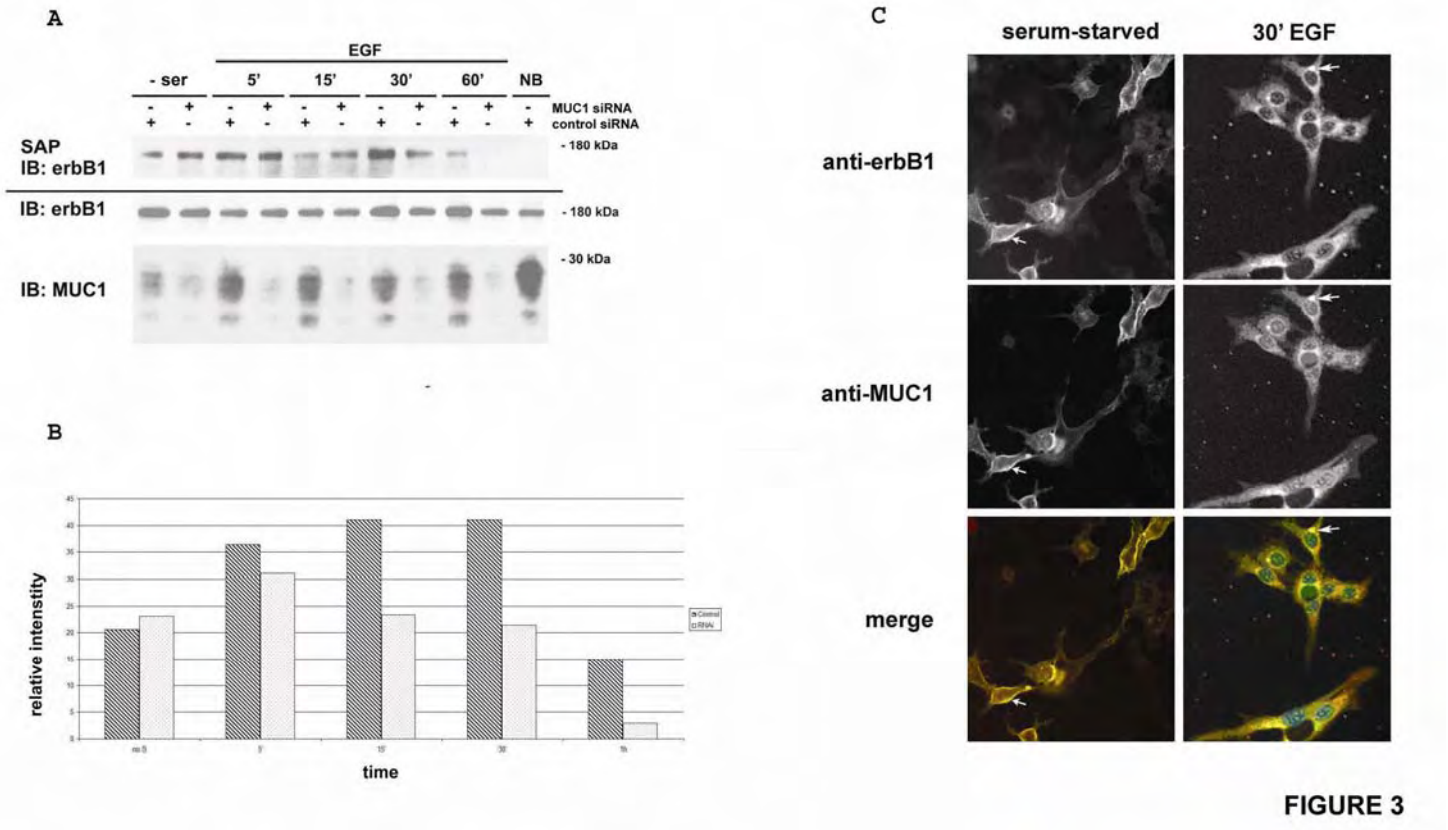
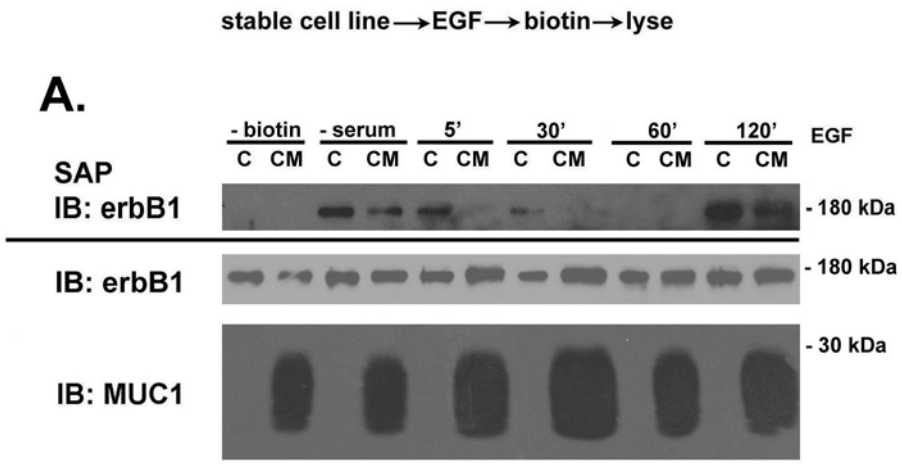

A
EGF
- ser
5'
15'
30'
60'
NB
MUC1 siRNA
control siRNA
SAP
IB: erbB1
- 180 kDa
IB: erbB1
- 180 kDa
IB: MUC1
- 30 kDa
B
relative intenstity
time
C
serum-starved
30' EGF
anti-erbB1
anti-MUC1
merge

FIGURE 3


stable cell line → EGF → biotin → lyse
A.
- biotin
- serum
5'
30'
60'
120'
EGF
C CM C CM C CM C CM C CM C CM
SAP
IB: erbB1
- 180 kDa
IB: erbB1
- 180 kDa
IB: MUC1
- 30 kDa

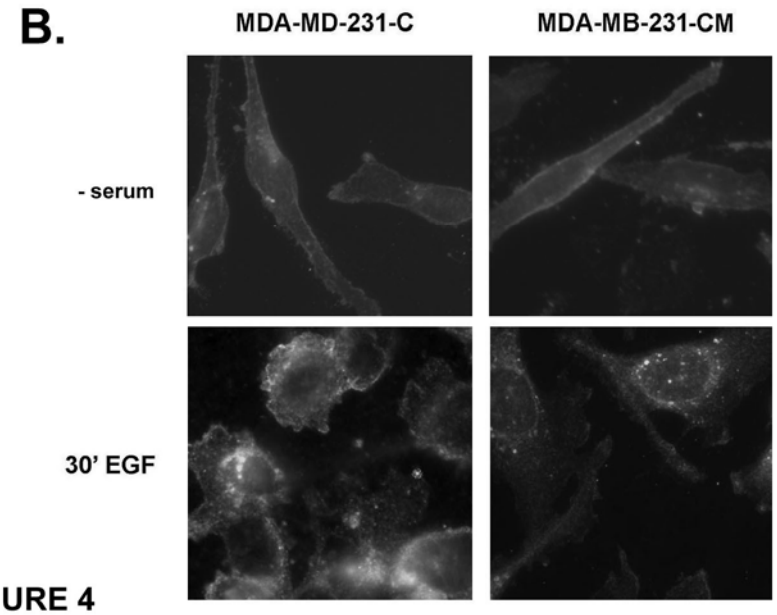

B.
MDA-MD-231-C
MDA-MB-231-CM
- serum
30' EGF

FIGURE 4

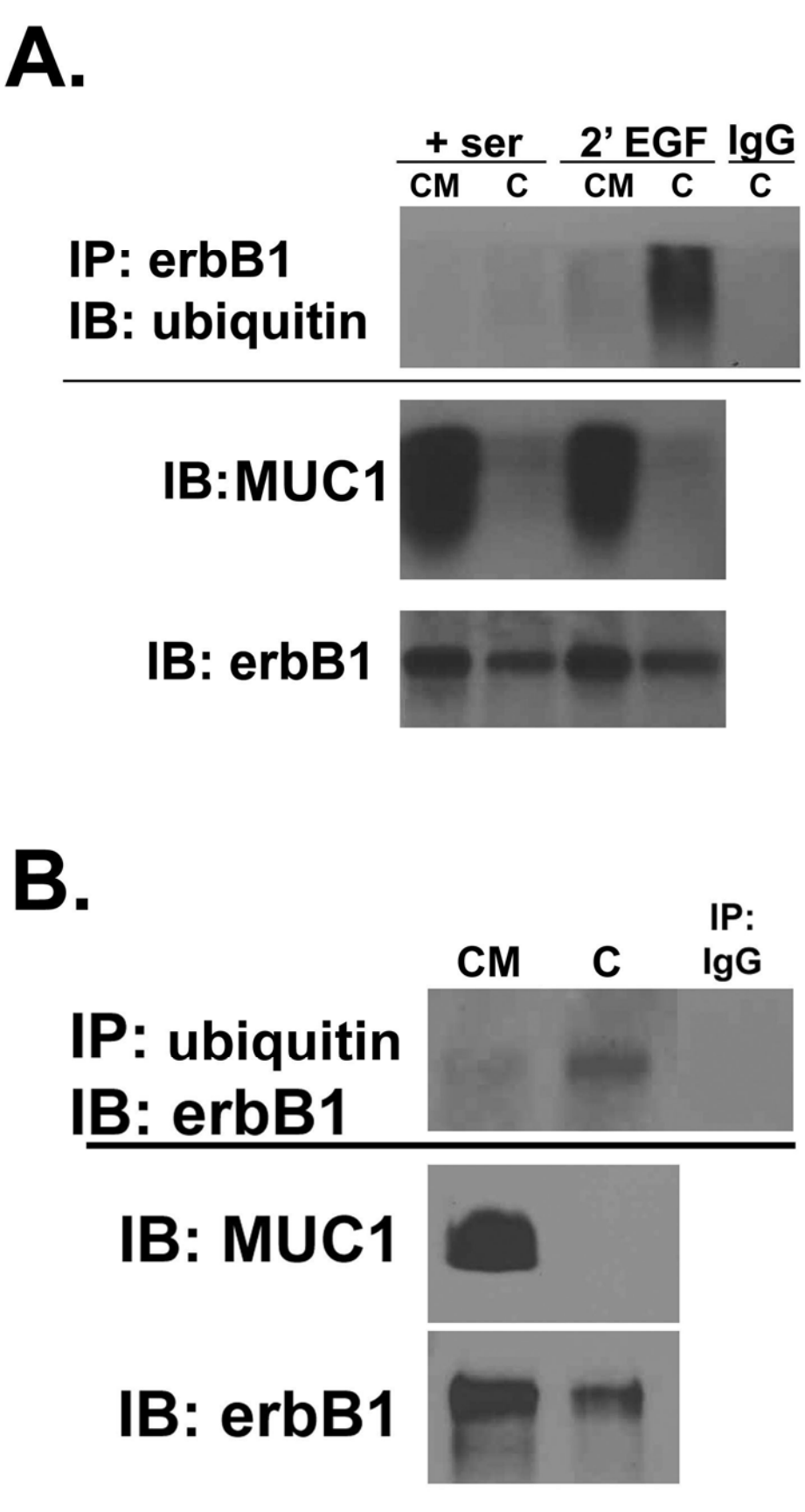

A.
+ ser
2' EGF
IgG
CM
C
CM
C
C
IP: erbB1
IB: ubiquitin
IB:MUC1
IB: erbB1
B.
IP:
CM
C
IgG
IP: ubiquitin
IB: erbB1
IB: MUC1
IB: erbB1

FIGURE 5

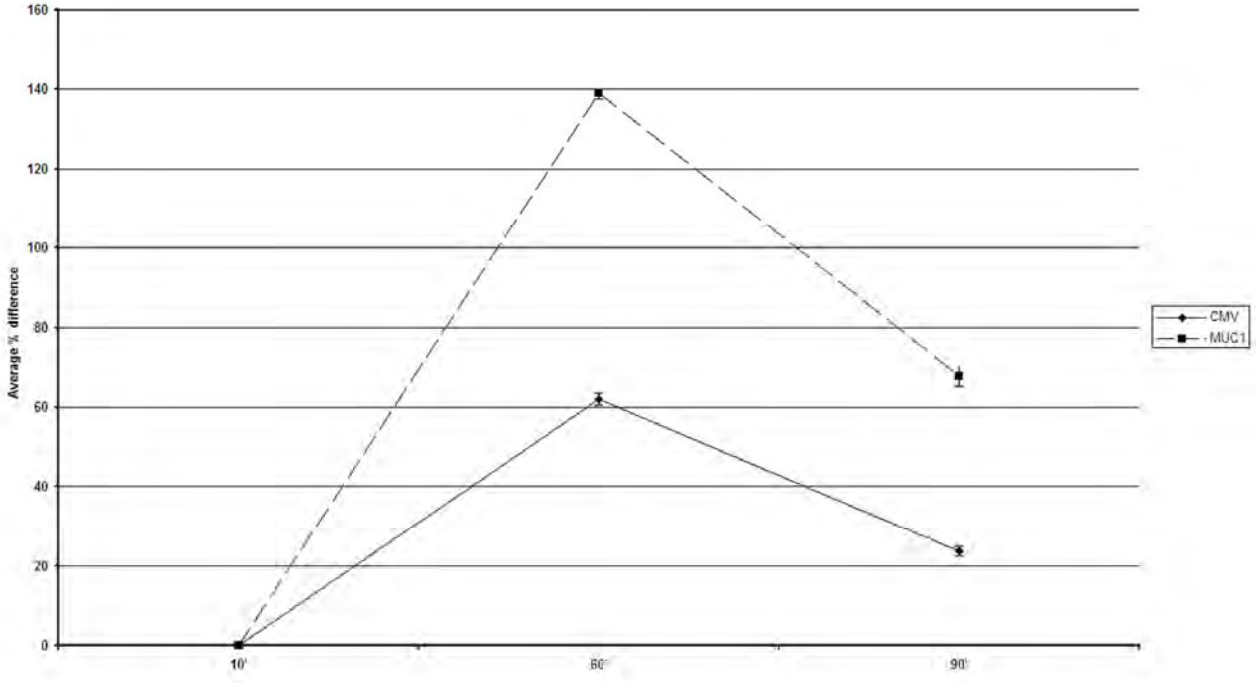

160
140
120
100
80
60
40
20
0
Average % difference
10'
60'
90'
CMV
MUC1

FIGURE 6